Analytical Methods

Simultaneous determination of main phenolic acids and flavonoids in tomato by micellar electrokinetic capillary electrophoresis

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Abstract

A methodology for the determination of tomato phenolic acids and flavonoids has been developed combining MEKC and DAD detection. The influence on polyphenol separation of pH and background electrolyte, BGE (borax, acetonitrile, methanol and SDS concentrations), was studied and optimized using response surface methodology and weighted desirability function. Separation of polyphenols was achieved within 20 min at 15 °C using 11.3 mM borax and 11.2 mM SDS adjusted to pH 8.5 as BGE. Validation was performed using standards and tomato extracts. Recoveries ranged from 77 to 106%. Acceptable repeatabilities were obtained for peak area (%RSD <3.1% and <3.7%) and migration times (%RSD <0.2% and <1.4%) for intra- and inter-day respectively. Detection limits ranged between 0.8 and 3.8 mg kg^{-1}. Five and seven of these polyphenols were determined in samples of tomato and related species. This methodology will be valuable tool in breeding programs, analyzing a large number of samples.

Keywords: Functional quality; MEKC; Plant breeding; Polyphenol; Response surface methodology

Chemical compounds studied in this article: Caffeic acid (PubChem CID: 689043); *p*-Coumaric acid (PubChem CID: 637542); *trans*-Ferulic acid (PubChem CID: 445858); Chlorogenic acid (PubChem CID: 1794427); Kaempferol (PubChem CID: 5280863); Quercetin (PubChem CID: 5280343); Myricetin (PubChem CID: 5281672); Naringenin (PubChem CID: 932); Rutin (PubChem CID: 5280805)

1 Introduction

Over the last decades, consumer demands in the food sector have changed, and consumers are increasingly conscientious of the contribution of food to their health (Siró, Kápolna, Kápolna, & Lugasi, 2008). In

the case of vegetables, health and nutritional value still scores relatively low in terms of importance during the purchase and consumption processes, but consumers with a high awareness of the relationship between food and health confer significantly more importance to these credence attributes (Ragaert, Verbeke, Devlieghere, & Debevere, 2004). In this context, more emphasis is placed in the development of breeding programs targeted to increase the concentration of bioactive compounds in vegetables (Goldman, 2011).

Although tomato does not stand out for its high concentration in bioactive compounds, the high levels of tomato intake around the World position it as one of the main sources of chemoprotective compounds in the diet (Chun et al., 2005). Although carotenoids and vitamin C are present in tomato at higher concentrations, polyphenols have gained attention during the last decade as key elements determining the functional value of tomato. Their high antioxidant capacity, may justify the correlation between antioxidant activity in tomato and the phenolic and flavonoid content (Ilahy, Hdider, Lenucci, Tlili, & Dalessandro, 2011). This activity may contribute to the prevention of oxidative damages, but in addition, plant phenolic compounds have been shown to inhibit the initiation, promotion and progression of cancer (Ramos, 2008). The action of quercetin, one of the prominent flavonoids in tomato has also been related with the inhibition and induction of survival and death signalling pathways (Stagos et al., 2012). In addition to reactive oxygen species (ROS) quenching, the antioxidant protective effect of flavonoids may be also related to their modulating activity of several detoxifying enzymes like lipoxygenase, cyclooxygenase, inducible nitric oxide synthase, monooxygenase, xanthine oxidase and NADH oxidase which are involved in cancer development (reviewed by Gibellini et al. (2011))

Main tomato fruit polyphenols are hydroxycinnamic acids, flavanones and flavonols (including glycosides), while anthocyanins are present only in certain varieties (Martí, Valcárcel, Herrero-Martínez, Cebolla-Cornejo, & Roselló, 2015; Martínez-Valverde, Periago, Provan, & Chesson, 2002; Mes, Boches, Myers, & Durst, 2008). Chlorogenic acid is the main hydroxycinnamic acid from tomato with concentrations ranging between 14 and 33 mg kg^{-1} fresh weight (fw) (Slimestad, Fossen, & Verheul, 2008). Lesser amounts of other hydroxycinnamic acids such as caffeic, *p*-coumaric and ferulic acids may be also found in cultivated tomatoes (Martínez-Valverde et al., 2002). Attending to flavonoids, naringenin chalcone can be found in tomato with concentrations up to 182 mg kg^{-1} fw (Slimestad et al., 2008). In addition, the flavanone naringenin can be found at lower concentrations, up to 13 mg kg^{-1} fw (Martínez-Valverde et al., 2002). The main flavonol in tomato is quercetin with a variable concentration between 7 and 44 mg kg^{-1} fw (Martínez-Valverde et al., 2002) in different tomato varieties. However, this compound is more frequently found in its glycosylated form as rutin, with concentrations up to 45 mg kg^{-1} fw (Slimestad et al., 2008) and its accumulation gives to the tomato peel its typical yellow colour. Other flavonols such as kaempferol and myricetin may be found as traces in cultivated tomato, though they are accumulated in related wild species (Martí et al., 2015; Martínez-Valverde et al., 2002; Shen, Chen, & Wang, 2007).

In the case of tomato, the development of breeding programs targeted to increase carotenoid concentration have been numerous (reviewed by Cebolla-Cornejo, Roselló, and Nuez (2013)). The emphasis placed in breeding programs for polyphenols has lagged behind carotenoids. Despite the limited variation in the primary gene pool (Colliver et al., 2002), wild species from the *Solanum* section *lycopersicum* have been used as sources of variation. Following this approach, tomato flavonoid content has been increased using the wild tomato species *Solanum pennellii* Correl (Willits et al., 2005).

One of the limitations of these types of programs is the necessity to evaluate a high number of individuals in segregant populations. Accordingly, it is necessary to rely on rapid and inexpensive analytical procedures to quantify prominent phenolic compounds in fruits and vegetables. Several chromatographic methods have been used to determinate polyphenols in food matrices, being the most widely used high-performance liquid chromatography (HPLC) coupled with UV–vis detection (Luthria, Mukhopadhyay, & Krizek, 2006; Martí et al., 2015) and/or mass (LC–MS) (Barros et al., 2012; De Paepe et al., 2013) or tandem mass spectrometry (LC–MS/MS) (Helmja, Vaher, Püssa, Raudsepp, & Kaljurand, 2008). An alternative to HPLC is constituted by electrodriven separation techniques, namely, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with UV detection. These methodologies constitute promising analytical tools for the routine determination of phenolic compounds in different types of food samples (Ehala, Vaher, & Kaljurand, 2005; Fukuji, Tonin, & Tavares, 2010; Helmja, Vaher, Gorbatsova, & Kaljurand, 2007; Herrero-Martínez, Oumada, Bosch, & Ràfols, 2007; Lee, Boyce, & Breadmore, 2012; Navarro, Núñez, Saurina, Hernández-Cassou, & Puignou, 2014). Main advantages of these methodologies are their high separation efficiency, high resolution power, shorter analysis times and low consumption of sample and reagents (its low consumption of reagents could classify CE as a green technique). Additionally, MEKC has a great advantage over CZE in the separation of mixture containing both ionic and neutral analytes. In MEKC, micelles of a surfactant (added to the running buffer at a concentration above its critical micellar concentration) form a pseudostationary phase. Thus, separation of neutral molecules is possible due to their different partition coefficients between aqueous and micellar phase. MEKC is usually carried out in non-coated fused silica capillaries with sodium dodecyl sulphate (SDS) as the most widely used surfactant. Thus, ionic micelles can also modify the selectivity in the separation of charged solutes via solute-micelle electrostatic and hydrophobic interactions. Despite these advantages, there are few reports (Helmja et al., 2007, 2008; Peng, Zhang, & Ye, 2008) dealing with the determination of phenolic compounds in tomato by these techniques.

The objective of this work is to develop a methodology specifically adapted to separate and quantify simultaneously prominent phenolic compounds from tomato. Since flavonoids such as rutin and others are difficult to be separated by CZE, the joint separation of phenolic acids and flavonoids was conducted by MEKC. For this purpose, background electrolyte (BGE) composition has been optimized using response surface methodology (RSM) to obtain the optimum separation conditions. The optimized methodology was successfully validated and applied to the analysis of different tomato samples.

2 Material and methods

2.1 Chemicals and reagents

The standards of all phenolic compounds investigated (caffeic acid, *p*-coumaric acid, *trans*-ferulic acid, chlorogenic acid, kaempferol, quercetin, myricetin, naringenin and rutin), as well as methanol (MeOH), acetonitrile (ACN) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (Syeinheim, Germany). Sodium hydroxide (NaOH), di-sodium tetra-borate 10-hydrate (borax) and SDS were from Panreac (Castellar del Vallés, Spain). Water was purified using a Milli-Q water system (Millipore, Molsheim, France). Uncoated fused-silica capillaries (67 cm total length, 60 cm effective length) with 375 µm od and 50 µm id were from Polymicro Technologies (Phoenix, AZ, USA). All stock solutions of polyphenols were prepared in MeOH/water (80:20 v/v) at 500 mg L^{-1} and were stored at −20 °C until their use. Working solutions at 20 mg L^{-1} were prepared by direct dilution of stock solutions in MeOH/water (48:52 v/v). All solutions were filtered through a 0.2 µm pore diameter PTFE filter before to be used.

2.2 Instrumentation and conditions

The CE experiments were performed with an Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (DAD). Before its first use, capillaries were rinsed at 50 °C with NaOH 1 M and 0.1 M for 5 min each one, followed by a rinse with water for 10 min. Prior to each working session, the capillary was flushed at 15 °C with NaOH 0.1 M during 5 min followed by BGE for 15 min. Between runs, capillary was flushed with 60 mM SDS during 3 min followed by BGE for 5 min. The injection was performed hydrodynamically at 7500 Pa for 20 s. The BGE was a prepared daily in an 11.3 mM borax buffer containing 11.2 mM SDS adjusted at pH 8.50. All solutions and buffers were filtered through a 0.22 µm cellulose acetate filter prior to injection. Separation procedure was performed at 25 kV at 15 °C. Detection and quantification was performed at different wavelengths depending on each polyphenol; 270 nm was employed for kaempferol, quercetin, myricetin, rutin and *p*-coumaric acid; 320 nm was used for naringenin, caffeic and ferulic acids and 345 nm was selected for chlorogenic acid. Each polyphenol spectrum was recorded using a DAD detector for later identification of compounds.

The effect of BGE composition was also examined to select the optimum composition and for tomato polyphenol separation. Factors analysed in the optimization study were borax concentration (from 2.5 to 15 mM), SDS concentration (from 0 to 60 mM), ACN concentration (from 0 to 20%) and pH (from 8.50 to 9.50). These ranges were based on preliminary studies. To avoid operational issues, the effect of temperature was also studied in a separate experiment (from 15 °C to 20 °C). The response variables employed for the optimization design were: resolution between two consecutive peaks, peak width and final time. A combined D-optimal design with experimental point determination by point exchange method (Anderson & Withcomb, 2005) was obtained and analysed with Design Expert Software (Version 9.0, Stat-Ease, Inc., Minneapolis, USA). The design consisted in 27 runs divided into three blocks. A combined regression model was adjusted and fittings to the data were checked with ANOVA. A weighted desirability function was used to determine the optimum separation conditions (targeted to maximize the separation of all the polyphenols, minimize the peak width and minimize final time) together with variable sized simplex algorithm (Anderson & Withcomb, 2005). The validity and adequacy of predicted separation model was verified with optimum separation conditions (five replicates) comparing predictions with observed values using a two sided *t*-test (α = 0.05).

2.3 Plant material and sample extraction

Tomato samples from four highly consumed cultivars (“Canario”, “Pera”, “Beef” and “Cherry”) were purchased in a local supermarket (Valencia, Spain). Accessions of a wild species related to tomato (*Solanum neorickii* D.M. Spooner, G.J. Anderson & R.K. Jansen and *Solanum pimpinellifolium* L.) were also used. A sample from the cv. “Kalvert” was also included. Samples were washed with tap water, homogenised in a blender and then kept frozen at −80 °C until analysis. *S. neorickii* and *S. pimpinellifolium* accessions were provided by the Genebank of the Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV, Spain). Wild materials were grown as reported by Leiva-Brondo Leiva-Brondo et al. (2012). Cv. “Kalvert” was grown in Navarra as specified in Lahoz et al. (2016a). The procedure described by Martí et al. (2015) was followed for polyphenol extraction. For the extraction, 1 g of homogenized sample was mixed with 5 mL of MeOH/water (48:52 v/v) containing 0.1% BHT (w/v). Then, samples were immersed in an ultrasonic bath Elmasonic S30H (Elma Electronics AG, Wetzikon Switzerland) at a frequency of 60 Hz during 177 min. All extraction procedure was performed in absence of light to avoid the oxidation of studied compounds. Finally, the resulting extracts were centrifuged at 4000 rpm (2361*g*) during 5 min in a refrigerated centrifuge at 4 °C, and supernatants were filtered through a 0.2 µm pore size PTFE filter before their injection in the CE system.

2.4 Method validation

A minimum of 6 levels were plotted for linear calibration, comprising concentrations between 1 ~~to~~ and 20 mg L^{-1}. Intra- and inter-day repeatabilities were evaluated using standards and tomato extracts. The LODs and LOQs were calculated for polyphenols present in tomato as the lowest concentration that provides a signal to noise ratio (S/N) equal to 3 and 10, respectively.

Real samples were also analysed using HPLC in order to provide a comparison with alternative methodology. For this purpose, the method and equipment described by Martí et al. (2015) was used.

3 Results and discussion

3.1 Optimization of MEKC conditions

The method described by Herrero-Martínez et al. (2007) was selected as a starting point to optimize the separation of polyphenols. This method enables the separation and quantification of several polyphenols in food samples, however it did not consider phenolic acids or other important flavonoids in tomato such as myricetin or rutin; therefore, our objective was to optimize it to provide a joint analysis of phenolic acids and flavonoids.

In a first step, different variations in the electrophoresis conditions were evaluated in order to identify the most important variables to consider. For this purpose, a standard mixture of the nine most important tomato polyphenols (20 mg L^{-1}) was used, including caffeic, *p*-coumaric, ferulic and chlorogenic acids, kaempferol, quercetin, myricetin, naringenin and rutin.

After this initial evaluation, it was concluded that borax concentration, SDS concentration, the use of an organic solvent and pH would play a prominent role. Herrero-Martínez et al. (2007) considered ACN and MeOH as modifiers in order to improve the resolution of polyphenols in real samples. In that case, the best compromise between resolution and analysis time was achieved using a BGE containing 10% MeOH. The high number of variables selected in our study required the selection of only one of these solvents. For this purpose, a second assay was developed. In this case, 5% of each organic solvent in a 25 mM borax buffer adjusted at pH 9.3 was used as BGE. In both cases, some of the peaks overlapped (Supplementary Fig. S1). However, in the case of ACN 7 peaks were distinguished, though two of them overlapped, while with MeOH, only 5 peaks could be identified. Therefore, the use of ACN provided a better resolution than MeOH of the problematic peaks, and it was selected as organic modifier for further optimization.

With all this information, a combined D-optimal design with experimental point determination by point exchange method was devised, including borax and SDS concentrations, ACN content and pH. Effective capillary length (60 cm) and separation temperature (20 °C) were kept as fixed factors.

Response surface models were developed in order to explain the influence of these factors on separation performance. All the factors studied were significant ($p < 0.01$) and the adjusted determination coefficients of the models (R^2adj) showed a good fit of the model for most of the response variables (Table 1). The models pointed out that SDS and ACN concentrations benefice the separation of polyphenols in an antagonistic way. Accordingly, the optimums included either SDS in the absence (or very low concentrations) of ACN or vice versa (Table 1). The optimums for pH were identified in the extremes (8.5 and 9.5) with lower resolutions for the pH values between them.

Table 1 Results from the optimization of BGE composition using RSM. Goodness of fit, significant factors identified, and best factor combinations are indicated.

		Final time	Peak resolution								Peak width								
			1–2[a]	2–3	3–4	4–5	5–6	6–7	7–8	8–9	1	2	3	4	5	6	7	8	9
Model R^2adjusted		0.96	0.08	0.55	0.20	0.05	0.10	0.78	0.97	0.60	0.67	0.65	0.70	0.63	0.86	0.89	0.64	0.81	0.72
Significant factors and interactions in the RSM models[b]		A, B, C, AB, AC, A^2	ND[c]	C, AC, BD	ND	ND	ND	A, AB, AC, A^2, C^2	A, C, D, AB, AD, BC, CD, A^2, B^2, C^2	B, AB, BC, CD, A^2	A, B, C	A, B, C, AB, BC	A, B, C, D, BC	B, C	A, B, C, BC	A, B, C, AB, BC, CD	B, AB, BC, CD	B, BC, CD, C^2	B, D, AB, BC, BD, CD, A^2, D^2
Best factor combination	ACN	0.0	20.0	20.0	0.0	0.0	ND	0.0	7.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0
	pH	9.5	ND	8.5	8.5	8.5	ND	9.3	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	9.5
	SDS	0.0	ND	0.0	0.0	0.0	0.0	0.0	35.0	60.0	0.0	57.0	0.0	0.0	0.0	5.0	3.0	30.0	33.0
	Borax	2.5	15.0	15.7	ND	15.0	ND	15.0	6.0	6.8	3.3	2.5	14.5	5.0	6.3	0.0	15.0	7.5	10.0

[a] Peak identification: 1 = rutin, 2 = naringenin, 3 = ferulic acid, 4 = *p*-coumaric acid, 5 = chlorogenic acid, 6 = kaempferol, 7 = myricetin, 8 = quercetin, 9 = caffeic acid.

[b] Model factors: A = borax (mM), B = SDS (mM), C = ACN (%), D = pH.

[c] ND = Not dependent.

Due to the complex interactions between all the studied factors, the determination of a single optimum combination was not an easy task because the maximum for each factor were not coincident (Fig. 1). A weighted desirability function was used to solve this problem. For this function, high weight (5 in a 5 point scale) was given to resolution between consecutive peaks and final time of analysis, and intermediate weight was assigned to peak width (3 in a 5 point scale). A BGE composition was recommended including 11.3 mM borax buffer, 11.2 mM SDS and pH 8.50 (conditions represented with a vertical line in Fig. 1). The reliability of the model was checked in a further

verification experiment. As shown in Table 2, no significant differences were found between the predicted and the real values for all optimized response factors: final analysis time, peak resolution and peak width.

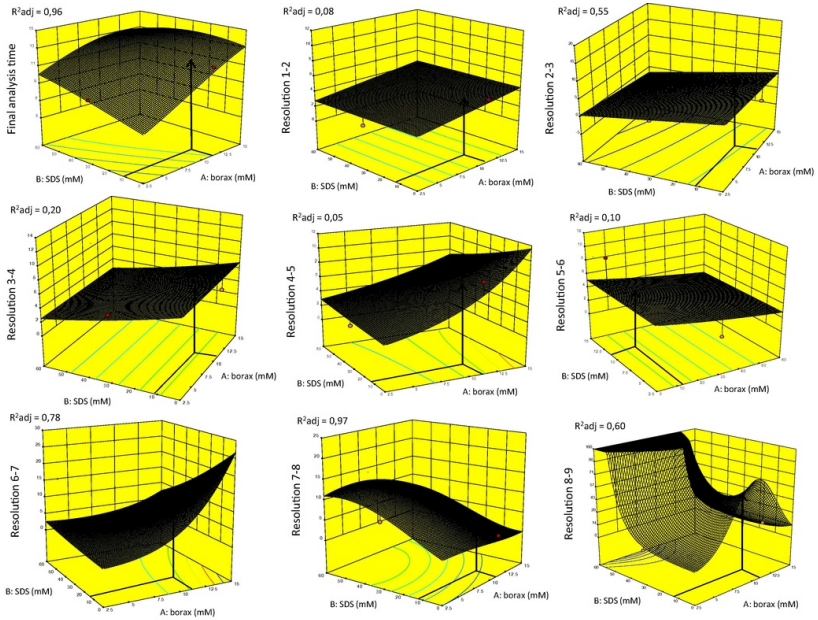

Fig. 1 Response surface plots of the main optimized variables (resolution between consecutive peaks and final analysis time) showing the effect of BGE composition (borax and SDS concentrations) at fixed conditions pH 8.5, 0% ACN. The selected BGE composition with the weighted desirability function is represented with a vertical black solid line. Peak identification: 1, rutin; 2, naringenin; 3, ferulic acid; 4, *p*-coumaric acid; 5, chlorogenic acid; 6, kaempferol; 7, myricetin; 8, quercetin; 9, caffeic acid.

Table 2 Results of predicted (mean ± SD), limits of 95% prediction interval and the real values (mean ± SD) for all optimized response factors.

Optimized parameter		Predicted	95% Prediction interval	Experimental
Final analysis time		10.3 ± 0.7	8.6–12.6	11.1 ± 0.2
Resolution between	Rutin-naringenin	3.6 ± 2.3	0–8.6	2.3 ± 0.1
	Naringenin-ferulic acid	4.4 ± 3.3	0–12.7	4.8 ± 0.2
	Ferulic acid-*p*-coumaric acid	6.1 ± 2.8	1.3–13.4	8.1 ± 0.1
	p-Coumaric acid-chlorogenic acid	4.2 ± 2.0	1.0–13.5	7.7 ± 0.1
	Chlorogenic acid-kaempferol	4.7 ± 2.7	1.0–15.2	6.1 ± 0.2
	Kaempferol-myricetin	2.0 ± 1.5	0–7.8	2.7 ± 0.1
	Myricetin-quercetin	3.7 ± 1.1	1.3–7.3	2.8 ± 0.1
	Quercetin-caffeic acid	44.1 ± 48.6	0.1–2223.8	22.3 ± 1.3
Peak width	Rutin	0.050 ± 0.014	0.024 – 0.094	0.054 ± 0.001
	Naringenin	0.063 ± 0.026	0–0.130	0.044 ± 0.001
	Ferulic acid	0.057 ± 0.017	0.025–0.117	0.043 ± 0.001

	p-Coumaric acid	0.062 ± 0.016	0.035–0.118	0.043 ± 0.001
	Chlorogenic acid	0.056 ± 0.012	0.032–0.093	0.058 ± 0.001
	Kaempferol	0.072 ± 0.014	0.043–0.115	0.055 ± 0.001
	Myricetin	0.113 ± 0.037	0.051–0.312	0.063 ± 0.002
	Quercetin	0.131 ± 0.043	0.057–0.419	0.104 ± 0.005
	Caffeic acid	0.098 ± 0.217	0–0.700	0.072 ± 0.001

The effect of temperature was then evaluated in order to further improve the resolution, with a range between 15 °C and 20 °C, by keeping the recommended BGE. The increased friction force at lower temperatures increased the time for analysis, but also improved the separation of close peaks (Fig. 2A). Once optimized the conditions for the analysis, the resulting MEKC method was validated.

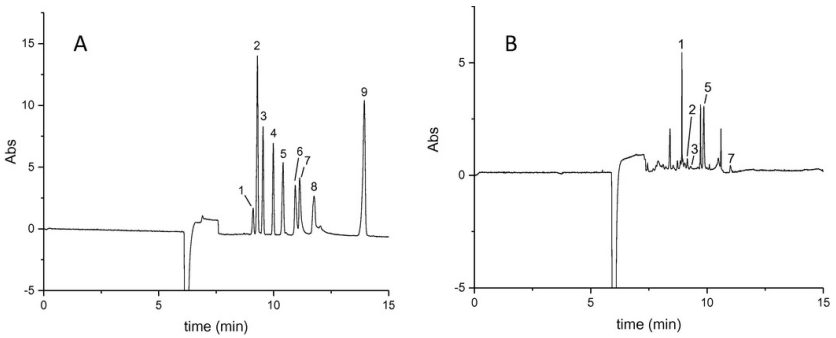

Fig. 2 Electropherograms of (A) standard mixture of polyphenols, (B) "Cherry" tomato. BGE: 11.3 mM borax, 11.2 mM SDS, pH 8.5. Voltage 25 kV, hydrodynamic injection 7500 Pa for 20 s, 15 °C. Capillary: 67 cm total length, 60 cm effective length, 375 μm od and 50 μm id. Peak identification: 1, rutin; 2, naringenin; 3, ferulic acid; 4, *p*-coumaric acid; 5, chlorogenic acid; 6, kaempferol; 7, myricetin; 8, quercetin; 9, caffeic acid.

3.2 Validation of MEKC method

Linearity, precision, sensitivity, LODs, LOQs and recoveries were evaluated employing polyphenol standards and "Canario" tomato as a real sample for the validation. Due to the variability in tomato genotypes, in the "Canario" sample used for validation not all the polyphenols were present and %RSD was calculated with fortified samples.

Excellent lineal regression models (r^2 > 0.995) were obtained for all studied polyphenols. Precision was calculated as intra- and inter-day repeatabilities (%RSD) of migration times and peak areas for standards and spiked tomato extracts at two fortification levels (12.5 and 50 mg kg^{-1}. Intra-day (n = 5) and inter-day (n = 2) precision of peak areas ranged from 1.7 to 3.1% and from 1.8 to 3.7%, respectively for standards, and from 0.2 to 4.0% and from 0.1 to 4.4%, respectively for spiked samples (Table 3). Compared to other methods employing similar CE techniques, these obtained %RSD values were similar or even lower (Arce, Ríos, & Valcárcel, 1998; Ehala et al., 2005; Herrero-Martínez et al., 2007). Moreover, compared to HPLC alternatives the obtained %RSD values were similar to those reported by other authors using DAD detection (Ribas-Agustí, Cáceres, Gratacós-Cubarsí, Sárraga, & Castellari, 2012) or even lower than those reported in HPLC-MS studies (De Paepe et al., 2013). Intra- (n = 5) and inter-day (n = 2) precision of migration times ranged from <0.1 to 0.2% and from <0.1 to 1.4%, respectively, for standards, and from <0.1 to 0.2% and from 0.3 to 1.7% respectively for spiked samples (Table 3). These values are similar to those reported by other authors (De Paepe et al., 2013).

Table 3 Analytical performance parameters of the optimized method using polyphenol standards and spiked tomato samples.

Compounds	Calibration equation y = ax + b			Intra-day repeatability[a] (n = 5) RSD (%)		Inter-day repeatability[a] (n = 2) RSD (%)		LOD; LOQ[b] (mg kg^{-1})	Tomato samples, Recovery (%), n = 5	
	a	b	r^2	t_m	Area	t_m	Area		Low (12.5 mg kg^{-1})	High (50 mg kg^{-1})
Caffeic acid	6.145	−2.403	0.9971	0.1; 0.2	2.3; 1.3	0.1; 0.9	2.9; 3.6	3.1; 10.3	106 ± 2	93 ± 1

p-Coumaric acid	3.988	−1.545	0.9950	0.1;<0.1	2.5; 0.8	0.6; 1.7	3.6; 0.1	1.6; 5.2	85 ± 4	98 ± 1
Ferulic acid	2.526	−0.602	0.9961	0.1;<0.1	2.4; 1.5	0.2; 0.3	3.7; 1.0	1.3; 4.3	101 ± 1	103 ± 2
Chlorogenic acid	3.378	−1.015	0.9971	<0.1; 0.1	3.1; 0.6	0.2; 0.7	3.7; 1.2	3.8; 12.6	101 ± 2	93 ± 1
Kaempferol	3.039	−2.614	0.9958	0.1; 0.1	2.5; 3.5	0.3; 0.9	1.8; 4.4	3.1; 10.2	103 ± 5	95 ± 4
Quercetin	3.241	−0.403	0.9985	<0.1; 0.1	2.3; 1.7	<0.1; 0.3	1.9; 3.5	3.3; 10.9	95 ± 3	86 ± 1
Myricetin	4.236	−2.546	0.9978	0.1; 0.1	1.8; 1.4	<0.1; 0.6	2.9; 2.4	2.5; 8.2	96 ± 1	83 ± 1
Naringenin	4.687	−0.388	0.9977	0.2; 0.1	2.4; 4.0	1.4; 0.6	2.2; 3.3	0.8; 2.6	77 ± 5	77 ± 4
Rutin	1.703	−0.116	0.9986	0.1;<0.1	1.7; 0.2	0.4; 0.3	2.9; 1.9	3.3; 11.1	96 ± 6	89 ± 0

[a] RSD obtained with: standards; tomato extracts.

[b] Detection and quantification limits obtained with tomato extracts.

As shown in Table 3, LODs ranged from 0.8 to 3.8 mg kg^{-1} fw. Other authors reported similar results working with CE techniques (Herrero-Martínez et al., 2007). As expected, due to the smaller optical path-length of CE detection cell compared to HPLC, LODs obtained are higher than those reported by other authors working with HPLC with DAD detection (Ribas-Agustí et al., 2012).

Recoveries were calculated in spiked tomato samples (average, n = 5) and ranged from 77 to 106% for the low fortification level (12.5 mg kg^{-1}), and from 77 to 103% for the high level (50 mg kg^{-1}). These results were comparable or even better than those reported by other authors (De Paepe et al., 2013; Ribas-Agustí et al., 2012; Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003).

3.3 Quantitation studies and application to real samples

The applicability of the MEKC method developed was evaluated analyzing five cultivated tomato samples representing different varietal types, and two wild tomatoes frequently used in breeding programs. Studied compounds were identified according their migration times and UV–vis spectra recorded with the DAD detector, by comparing these data with those obtained with standards. In all cases, peak purity values were higher than thresholds.

As shown in Table 4 and Fig. 2B, five of the studied phenolic compounds could be determined in the tomato samples analysed. It should be considered that in the development of the method we included 9 phenolic compounds described at any time in tomato, but the occurrence of some of them is very rare or are present as traces.

Table 4 Determination of main polyphenols (mg kg^{-1} fw) in tomato and tomato wild relatives (mean ± SD).

Sample	Method	Caff[a]	*p*-Cou	Fer	Chlor	Kaem	Quer	Myr	Naring	Rut
"Canario"	MEKC	[b]			8.74 ± 0.31[c]				6.15 ± 0.24	7.63 ± 0.05[c]
	HPLC	1.18 ± 0.01	0.86 ± 0.01	1.06 ± 0.01	9.47 ± 0.01				8.07 ± 0.06	5.43 ± 0.03
	RSD (%)	–	–	–	6	–	–	–	19	24
"Pera"	MEKC				5.89 ± 0.21[c]				3.07 ± 0.12	27.37 ± 0.00
	HPLC	0.96 ± 0.01			6.85 ± 0.22				3.59 ± 0.10	21.43 ± 0.16
	RSD (%)	–	–	–	11	–	–	–	11	17
"Cherry"	MEKC			1.59 ± 0.06[c]	23.51 ± 1.03			3.35 ± 0.08[c]	4.20 ± 0.12	30.92 ± 1.36
	HPLC	1.54 ± 0.01	1.55 ± 0.00	1.31 ± 0.03	19.56 ± 0.08			2.62 ± 0.03	3.81 ± 0.03	24.98 ± 0.24
	RSD (%)	–	–	14	13	–	–	17	7	15

"Kalvert"	MEKC				17.86 ± 0.31				7.93 ± 0.31	43.58 ± 0.41
	HPLC	1.34 ± 0.03	0.86 ± 0.00	0.80 ± 0.02	15.62 ± 0.36				9.99 ± 0.17	35.00 ± 0.03
	RSD (%)	–	–	–	9	–	–	–	16	15
"Beef"	MEKC				5.25 ± 0.10[c]					4.07 ± 0.22[c]
	HPLC	0.94 ± 0.02		0.86 ± 0.01	5.68 ± 0.11					3.11 ± 0.08
	RSD (%)	–	–	–	6	–	–	–	–	19
S. pimpinellifolium	MEKC	3.81 ± 0.61[c]		3.57 ± 0.20[c]	264.21 ± 2.08			3.70 ± 0.02[c]	5.70 ± 0.12	33.61 ± 0.41
	HPLC	4.43 ± 0.20	1.32 ± 0.04	2.78 ± 0.07	307.07 ± 1.30			2.67 ± 0.09	4.80 ± 0.01	32.18 ± 0.06
	RSD (%)	11	–	18	11	–	–	23	12	3
S. neorickii	MEKC	5.21 ± 0.03[c]	9.68 ± 0.48	1.97 ± 0.13	152.02 ± 1.65			5.34 ± 0.14[c]		92.47 ± 2.25
	HPLC	5.86 ± 0.11	7.90 ± 0.17	2.16 ± 0.00	191.32 ± 1.96	1.81 ± 0.03		5.84 ± 0.09		93.21 ± 0.80
	RSD (%)	8	3	7	16	–	–	6	–	1

[a] Caffeic acid (caff), *p*-coumaric acid (*p*-cou), ferulic acid (fer), chlorogenic acid (chlor), kaempferol (kaem), quercetin (quer), myricetin (myr), naringenin (naring), rutin (rut).

[b]

[c] Under Limit of Quantification. Values included to test the capabilities of the method under low concentrations and only to provide a comparison with HPLC determination.

The samples were also analysed with HPLC in order to provide a comparison with previous alternative methodologies. When the concentration of polyphenols was very small, they could be quantified with HLPC, but these remained under LOD with the MEKC analysis. In this cases, small peaks could be observed, but no comparison could be provided. On the other hand, when the areas of peaks detected with MEKC were lower than LOQs, concentrations were calculated in order to provide a comparison. Considering all the pairs quantified with both methodologies, 39% of the comparisons showed %RSD lower than 10%, another 39% between 11% and 15%, 18% between 15% and 20% and a 4% between 20% and 24%. In 40% of the determinations with %RSD higher than 15% the quantification was made under LOQ, and thus a higher %RSD was expected.

Chlorogenic acid and rutin were the most abundant polyphenols found in the samples analysed. As expected, within the cultivated species "Cherry" tomatoes showed the highest levels chlorogenic acid (23.51 mg kg^{-1} fw), whereas "Canario" and "Pera" tomatoes did not reach half this value (8.74 and 5.89 mg kg^{-1} fw respectively). Since polyphenols are mainly accumulated in the surface of tomato (Torres, Davies, Yañez, & Andrews, 2005), smaller tomatoes have higher surface to volume ratio, consequently the accumulation of polyphenols would also be higher in this type of materials. In fact, the development of new tomato varieties with outstanding functional value are targeted to the combination of *high pigment* (*hp*) genes and the restoration of anthocyanin accumulation in the fruit, specifically in "Cherry" varieties (Sestari et al., 2014). The cv. "Kalvert" showed high levels of chlorogenic acid (17.86 mg kg^{-1} fw) and rutin (43.58 mg kg^{-1} fw). This is a high lycopene cv. that probably carries a *hp* mutation (Lahoz et al., 2016b), and even being quite bigger than a "Cherry" tomato, it presents similar levels of polyphenols. It even doubled the contents of varieties with similar size. Increased levels of polyphenols (including cholorogenic and quercetin, the precursor of rutin) have been previously associated with *hp* genes (reviewed by Martí, Roselló, & Cebolla-Cornejo, 2016), thus confirming that this cv. might carry an *hp1* or *hp2* mutation.

Martínez-Valverde et al. (2002) also found chlorogenic acid as the main hydroxycinnamic acid in different tomato varieties with values up to 32 mg kg^{-1} fw. However, in this work, the "Cherry" variety was not included. Raffo, La Malfa, Fogliano, Maiani, and Quaglia (2006) also confirmed the prevalence of chlorogenic acid in "Cherry" tomatoes, with the highest contents of 54.4 mg kg^{-1} fw found in April harvests that decline in July harvests (our case) down to 31.2 mg kg^{-1} fw.

The potential of wild species as sources of variance in breeding programs was confirmed by our results, as they triplicated in the worst case the contents of the best cultivated material. Among them, *S. pimpinellifolium* presented the highest concentrations of this acid: 264.21 mg kg^{-1} fw. Wild species related with tomato have been used previously by breeders in order to increase the levels of flavonoids (Willits et al., 2005). In that case, *Solanum pennellii* Correl was used to restore quercetin accumulation in the flesh. In our case, the high accumulation of chlorogenic acid in the accession of *S. pimpinellifolium* could be used to increase this hydroxycinnamic acid as well.

In tomato, the second most abundant hydroxycinnamic acid is caffeic acid. Martínez-Valverde et al. (2002) found variable accumulations, between 1.39 and 13 mg kg^{-1} fw, depending on the variety considered. In that study, ferulic and *p*-coumaric acids presented lower concentrations. In "Cherry" tomatoes Raffo et al. (2006) did not find important differences between caffeic and *p*-coumaric, while ferulic showed lower concentrations. In our case, in the cultivated tomato samples caffeic and p-coumaric acids were under LODs with MEKCand only small quantities could be detected with HPLC, whereas ferulic acid was found at 1.59 mg kg^{-1} fw only in "Cherry". On the other hand, the highest quantities of hydroxycinnamic acids were found in *S. neorickii*.

Contents of rutin (Table 4) were also higher in "Cherry" tomatoes (30.92 mg kg^{-1} fw) than in "Canario" (7.63 mg kg^{-1} fw), "Beef" (4.07 mg kg^{-1} fw) and "Pera" (23.37 mg kg^{-1} fw), but lower than cv. "Kalvert" (43.58 mg kg^{-1} fw). This time the accessions from wild species did not provide outstanding values when compared to "Cherry" tomatoes. The accession from *S. neorickii* gave the highest level of rutin, 92.47 mg kg^{-1} fw, higher than "Cherry", but maybe not enough to justify its use in breeding programs.

Naringenin contents ranged from traces in "Beef" to 7.93 mg kg^{-1} fw in "Kalvert", although it was not present in *S. neorickii* sample (Table 4). This fact was also expected, since in tomato, the typical yellowish color of the peel is due to the accumulation of naringenin chalcone (Ballester et al., 2010), and this trait is not present in the green fruit of *S. neorickii.*

The flavonols kaempferol and quercetin were not detected in the analysed samples. Only low values of kaempferol were detected with HPLC. Myricetin was found at concentrations lower than the quantification limit for "Cherry" *S. pimpinellifolium* and *S. neorickii*. Raffo et al. (2006) did not consider these compounds in the evaluation of "Cherry" tomato polyphenols, while Martínez-Valverde et al. (2002) analysed hydrolyzed samples. In that study, only traces of kaempferol could be identified in some varieties, while most quercetin may have arisen from the hydrolysis of rutin. Other studies, have also failed to detect these flavonols as free aglycones at the red ripe stage (Martí et al., 2015).

4 Conclusion

This work describes a rapid and easy MEKC procedure for the determination of nine tomato polyphenols capable to analyze a high number of samples typical of quality control and breeding programs. The method developed fulfills the requirements for the analysis of polyphenols presents in tomato using capillary electrophoretic methodologies. The BGE composition has been optimized using RSM and weighted desirability function, using tomato as a model. The best separation results were obtained at 15 °C using a micellar BGE without any organic modifier; enabling the determination of the nine phenolic compounds in 20 min. The differences within the response surfaces obtained could be due to a high level of interaction between factors, therefore it is mandatory to reach a compromise solution to maximize the separation in a global perspective.

The repeatability, LODs and recoveries in tomato samples are similar to the results of other authors working with similar or more sensitive techniques. Moreover, the applicability of the method has been checked using three commercial tomato cultivars and two wild tomato relatives. These results highlight the usefulness of the method developed for its application to the determination of tomato polyphenols in quality controls or in the development of breeding programs.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.10.105.

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Appendix A. Supplementary data

Multimedia Component 1

Supplementary Fig. S1

Highlights

- MEKC-DAD method for the determination of nine tomato polyphenols in 20 min.
- BGE used consisted in a 11.3 mM borax buffer containing 11.2 mM SDS adjusted to pH 8.5.
- Separation of polyphenols was optimized using RSM and weighted desirability function.
- It was applicable to high throughput analysis typical from breeding programs.

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